Effect of Inoculum Preparation and Dietary Energy on Microbial Numbers and Rumen Protein Degradation Activity

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ABSTRACT

Chilling of whole rumen contents prior to preparation of strained rumen fluid enriched with particle-associated microorganisms resulted in rumen inoculum with the highest microbial dry weight, total bacterial counts, and degradation rates for casein and soybean meal. Strained rumen fluid, whole rumen contents blended with strained rumen fluid, chilled strained rumen fluid, and strained rumen fluid plus particle-associated microorganisms were lower in bacterial counts and microbial dry weight. Except for strained rumen fluid plus particle-associated microorganisms, protein degradation rates were also lower. Three ruminally cannulated cows were used in a 3 × 3 Latin square experiment to determine the effect of diet on rumen microbial numbers and protein degradation rates. Cows were fed the following diets ad libitum: 1) 100% alfalfa hay (20.7% crude protein); 2) 63% alfalfa hay and 37% corn-soybean meal concentrate (18.2% crude protein); and 3) 37% alfalfa hay and 63% corn-soybean meal concentrate (15.1% crude protein). Diet 3 yielded rumen contents with the highest concentration of microbial dry weight, total and viable bacterial counts, total protozoal counts, and fractional degradation rates for casein and bovine serum albumin. However, degradation rate per unit microbial dry matter was not altered by diet.

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INTRODUCTION

An understanding of the influence of diet on the rumen microbial population may aid in discovery of how to decrease rumen protein degradation. Researchers have suggested that rumen microorganisms may adapt to a protein source in the diet and increase the protein degradation rate. On a fresh alfalfa diet, compared with a hay and concentrate diet, rumen proteolysis of fraction 1 leaf protein increased three- to ninefold after an adaptation period (19). Dietary protein source appears to influence its degradability (16, 17) and on microbial adaptability (22). Hazelwood et al. (14) found an increase in the proportion of proteolytic *Streptococcus bovis* in the rumen of cattle fed a fresh forage diet compared with those fed a dry diet.

Studies on the effect of diet on rumen microbial proteolytic activity in vitro should be based on representative samples of rumen microbes. Most rumen inocula are prepared from only the liquid fraction of rumen contents, whereas 70 to 80% of the total microbial mass was associated with the particulate fraction (9, 13). Craig et al. (10) have compared methods for enriching in vitro inocula with particle-associated organisms.

These studies were designed to determine the effect of rumen inoculum preparation method and the influence of dietary energy concentration on microbial numbers and protein degradation activity.

MATERIALS AND METHODS

Inoculum Preparation Experiment

Rumen Contents. Rumen contents were obtained approximately 4 h after feeding from a ruminally cannulated lactating Holstein cow fed

a diet of alfalfa hay, corn silage, corn grain, and soybean meal (SBM). Rumen inocula were prepared in five different ways: 1) whole rumen contents were strained through two layers of cheesecloth (strained ruminal fluid, SRF); 2) equal volumes of whole rumen contents (WRC) and SRF blended (B) for 1 min at high speed in a Waring blender and then strained through four layers of cheesecloth (WRC plus SRF, B); 3) SRF prepared from whole ruminal contents chilled 4 h (SRF, 4°C); 4) equal volumes of strained rumen fluid and particle-associated microorganisms (PO) extracted by washing the residual solids with McDougall's buffer (18) and squeezing first through two layers then eight layers of cheesecloth (10) (SRF plus PO); and 5) SRF plus PO prepared the same as (10) except the whole ruminal contents were chilled 4 h prior to inoculum preparation (SRF plus PO, 4°C).

Dietary Energy Concentration Experiment

Animals and Feeding. Three lactating Holstein cows fitted with rumen cannulae were randomly assigned to three dietary treatment sequences in a 3 × 3 Latin-square design (Table 1). Diets were initially balanced to be isonitrogenous, but alfalfa hay was higher in protein than expected (20.7% CP; Table 1). Cows were fed ad libitum four times daily and water was continually available. Cows were allowed a 14-d adjustment period followed by a 14-d sampling period. Rumen contents were transferred between cows when switching diets to reduce the time for microbial adaptation.

Rumen Contents. Rumen contents were collected approximately 4 h after feeding. Method 5 [SRF plus PO, 4°C (10)] was used to determine microbial numbers and protein degradation activity.

Rumen Parameters. Rumen pH was measured immediately after collection and straining of rumen contents through two layers of cheesecloth. One milliliter of 50% (vol/vol) H_2SO_4 was then added to 50 ml of SRF and stored (−20°C) until ammonia and VFA analysis. Ammonia was determined colorimetrically by the phenol-hypochlorite method (6) and VFA by gas chromatography using a column packing of SP-1200/H_3PO_4 on Chromosorb W AW (20). An internal standard, 2-ethylbutyrate, was used. Samples were collected on 3 separate d during each period and were analyzed in duplicate.

Microbial Numbers. Microbial counts were determined in duplicate on 3 separate d in the SRF plus PO, 4°C inoculum according to the procedure of Mahadevan et al. (16). Bacteria were counted in a hemacytometer (AO Scientific Instruments, Buffalo, NY), and the protozoa were counted in a 49.75 × 19.75 × 1-mm counting chamber. A Nikon biological microscope (Tokyo, Japan) was used at 1600× oil-immersion phase contrast for bacteria and 160× bright field for protozoa. Viable counts were determined on medium 10 (8) with the following modifications: a 2.5% cysteine-HCl-H_2O solution prepared under CO_2 was added as a reducing agent at 2 ml/100 ml of medium 10; cysteine-HCl-H_2O and sodium carbonate were added before autoclaving. The plates were

TABLE 1. Diet compositions.[1]

Item	100F	63F/37C	37F/63C
		(% of dry matter)	
Alfalfa	100	63	37
Concentrate	0	37	63
Corn	...	35.1	54.0
Soybean meal	...	1.5	7.3
Vitamin and mineral mix	...	.5	1.6
Crude protein	20.7	18.2	15.1
Neutral detergent fiber	47.6	36.6	31.2
Acid detergent fiber	31.6	21.4	14.2
Ash	10.3	8.2	5.9

[1] Percentage of the diet as alfalfa hay (F) or from concentrate (C).

poured in a glove box (Coy Laboratory Products Inc., Ann Arbor, MI) with an atmosphere of 85% N_2, 10% CO_2, and 5% H_2. The rumen inocula were diluted in anaerobic dilution solution (7). Using a repeating pipet, plates were inoculated in quadruplicate at two different dilutions per inoculum source using a sterile, bent glass rod. Plates were incubated at 39°C for 5 d in the glove box incubator.

Microbial Dry Weight and Nitrogen Content. Dry matter concentration of each inoculum was determined in quadruplicate by centrifugation (31,000 × *g*, 15 min, 2°C) of 10-ml aliquots. Supernatants were discarded and pellets dried for 48 h at 60°C. Nitrogen content of pellets was determined by Kjeldahl analysis (1).

Protein Degradation Activity. Protein sources [casein and bovine serum albumin (BSA)], .500 g of each, were weighed into spinner flasks (Bellco, Vineland, NJ) and dissolved in 100 ml of McDougall's buffer (18) at 39°C. A composite sample was prepared from five different solvent-extracted SBM and had a N solubility of 30% in McDougall's buffer. An amount (1 g) of this sample to provide equal N concentration to the other protein sources was weighed into spinner flasks and soaked in 100 ml McDougall's buffer at 39°C. The SBM was used in the inoculum preparation experiment only while casein and BSA were used in both experiments. Rumen inocula were warmed to 39°C and 1.5 m*M* hydrazine sulfate (4), 3.0 m*M* 2-mercaptoethanol, and 1.0% maltose were added and dispensed under CO_2 at 200 ml per spinner flask. Incubations were carried out at 39°C, and samples were removed from each flask in duplicate under CO_2 at .5, 1, 1.5, 2, 2.5, 3 h (plus 4 h for the inoculum preparation experiment). Incubations were terminated by the addition of 1 ml 2% (wt/vol) cetyltrimethylammonium bromide to 1-ml samples followed by mixing and freezing. Blank flasks with no added protein source were also included with each experiment for each inoculum source. Single blank and plus protein flasks were used in the inoculum preparation study and duplicate flasks were used in the feeding experiment. Concentrations of total free amino acids and ammonia were determined in sample supernatants (10,000 × *g*, 15 min, 4°C) including correction for recoveries of leucine and ammonia standards (6). Fractional protein degradation rates were computed from the net release of total free amino acids and ammonia (5). Protein degradation rates were also computed on the basis of amount degraded per unit microbial DM. Experiments were run on 3 different d per treatment sequence.

Chemicals. Cysteine-HCl-H_2O, casein, BSA, hydrazine sulfate, 2-mercaptoethanol, and maltose were from Sigma Chemical Company, St. Louis, MO.

Statistical Analysis. Data were statistically analyzed by one-way analysis of variance with mean separation accomplished by the least significant difference method after a treatment effect (at least $P<.05$) was detected (23).

RESULTS AND DISCUSSION

Inoculum Preparation Experiment

The inoculum preparation experiment compared results from using inocula enriched with particulate microbes with those obtained using the methods of other workers. The effect of inoculum preparation method on microbial mass and numbers is in Table 2. Microbial dry weight and total bacterial count were highest with the chilled SRF plus PO followed by the unchilled SRF plus PO. Chilled SRF plus PO, and blended WRC plus SRF yielded the highest viable bacterial counts. Dehority and Grubb (11) found that chilling rumen contents on ice for 6 h before blending increased the viable bacterial counts by 50%. Blending whole rumen contents increased the viable bacterial count by approximately 30% over SRF (15). Blended WRC plus SRF had the highest percent viability, possibly due to the breakage of clumps with blending. Total protozoal counts were highest with the unchilled SRF plus PO and lowest on the blended WRC plus SRF. Senshu et al. (21) reported that blending caused lysis of protozoa.

In a separate experiment, the effect of inoculum preparation method on protein degradation activity was determined. Viable counts were not determined in this experiment. Microbial dry weight and total bacterial counts were again highest on the chilled SRF plus PO inoculum (Table 3). Total protozoal counts were highest with the chilled SRF plus PO treatment. The higher protozoal counts after chilling in this experiment possibly resulted from rumen contents being warmed too rapidly in the pre-

TABLE 2. Effect of inocula preparation method on microbial numbers.

Rumen inocula[1]	Microbial dry weight	Microbial N	Total bacterial count	Viable bacterial count	Viability	Total protozoal count
	(mg/ml)	(% DMB[2])	(× 10^9/ml)	(× 10^9/ml)	(%)	(× 10^4/ml)
SRF	16.24[d]	7.69[b]	8.38[c]	1.77[c]	21.37[c]	16.96[c]
WRC + SRF, B	15.18[d]	7.79[a,b]	9.50[c]	3.04[a]	32.05[a]	5.43[c]
SRF, 4°C	20.41[c]	8.04[a,b]	9.34[c]	2.31[b]	25.10[b]	12.89[c]
SRF + PO	23.10[b]	7.65[b]	12.33[b]	2.18[b,c]	17.69[d]	46.14[a]
SRF + PO, 4°C	26.40[a]	8.13[a]	15.67[a]	3.35[a]	21.40[c]	31.21[b]

[a,b,c,d]Means within columns with different superscripts differ ($P<.05$).

[1]SRF = Strained ruminal fluid; WRC + SRF, B = equal volumes of whole rumen contents and SRF blended; SRF, 4°C = SRF prepared from whole ruminal contents chilled 4 h; SRF + PO = equal volumes of strained rumen fluid and particle-associated microorganisms (PO) extracted by washing the residual solids with McDougall's buffer and squeezing first through two layers, then eight layers of cheesecloth; SRF + PO, 4°C = SRF + PO chilled 4 h prior to inoculum preparation.

[2]DMB = Dry matter basis.

TABLE 3. Effect of inocula preparation method on protein degradation activity.

Rumen inocula[1]	Microbial dry weight	Microbial N	Total bacterial count	Total protozoal count	Degradation rate			Fractional degradation rate		
					BSA[2]	SBM[2]	Casein	BSA	SBM	Casein
	(mg/ml)	(% DMB[2])	(× 10^9/ml)	(× 10^4/ml)	(mg N/h per g DM)			(h^{-1})		
SRF	13.88[d]	7.90	7.92[c]	7.67[c]	.649[b]	.921[b]	1.390[b]	.036[b]	.055[c]	.081[c]
WRC + SRF, B	13.09[d]	8.02	9.38[c]	2.04[d]	.352[c]	.754[b]	1.626[b]	.018[c]	.043[c]	.089[c]
SRF, 4°C	17.72[c]	7.86	9.58[c]	8.55[b,c]	.925[a]	.951[b]	1.363[b]	.065[a]	.073[b]	.101[c]
SRF + PO	23.72[b]	7.96	17.17[b]	9.63[b]	.775[a]	1.398[a]	2.262[a]	.073[a]	.143[a]	.225[b]
SRF + PO, 4°C	26.00[a]	7.96	19.25[a]	12.08[a]	.759[a]	1.372[a]	2.419[a]	.078[a]	.154[a]	.263[a]

[a,b,c,d]Means within columns with different superscripts differ ($P<.05$).

[1]SRF = Strained ruminal fluid; WRC + SRF, B = equal volumes of whole rumen contents and SRF blended; SRF, 4°C = SRF prepared from whole ruminal contents chilled 4 h; SRF + PO = equal volumes of strained rumen fluid and particle-associated microorganisms (PO) extracted by washing the residual solids with McDougall's buffer and squeezing first through two layers, then eight layers of cheesecloth; SRF + PO, 4°C = SRF + PO chilled 4 h prior to inoculum preparation.

[2]DMB = Dry matter basis; BSA = bovine serum albumin; SBM = soybean meal.

vious experiment, which may have promoted protozoal lysis (B. A. Dehority, personal communication).

Protein degradation rates based on microbial DM and fractional degradation rates were determined using three protein substrates: casein, SBM, and BSA (Table 3). Chilled SRF plus PO gave the highest casein degradation rates followed by unchilled SRF plus PO. Chilled and unchilled SRF and blended WRC plus SRF all had casein degradation rates, both per unit DM and fractional rates, that were about half those for SRF plus PO. Enriching inoculum with PO (inocula SRF plus PO and SRF plus PO, 4°C; Table 3) also yielded the highest protein degradation rates for SBM. Results with BSA were not as clear cut; BSA degradation rates were similar for the chilled SRF plus PO, unchilled SRF plus PO, and chilled SRF.

These results emphasize the importance of microbial extraction methods for obtaining representative rumen inocula for in vitro experiments. Chilled SRF plus PO yielded the greatest microbial dry matter and bacterial numbers and tended to have the highest protein degradative activity. The particulate fraction of rumen contents may contain as much as 75% of the proteolytic activity (3). Chilling and washing rumen digesta particles with buffer appear to enhance the removal of microorganisms associated with the particulate phase (10). Therefore, some method to enrich inocula with particle-associated microorganisms is essential during in vitro experiments.

Dietary Energy Concentration Experiment

Rumen parameters from the dietary energy concentration experiment are in Table 4. Increasing dietary concentrate decreased rumen pH. Rumen ammonia was higher on the diets containing concentrate (diets 63F/37C and 37F/63C; Table 4) even though these diets were lower in crude protein than the 100% forage diet. As expected, total VFA was greatest on the 63% concentrate diet, as were the molar proportions of propionic and butyric acid. Isovaleric plus 2-methylbutyric acids, which are formed from catabolism of leucine and isoleucine, were also elevated on the 37% forage diet. As expected, the 100% forage diet had the highest molar proportion of acetic acid and the lowest molar proportion of propionic acid.

The effects of dietary energy concentration on microbial numbers and protein degradation activity are in Table 5. There were approximately 15 times more total protozoa on the 37% forage diet compared with the 100%

TABLE 4. Effect of diet on feed intake and rumen parameters.

Item	Diet[1]			SE
	100F	63F/37C	37F/63C	
Dry matter intake, kg/d	16.9	19.4	15.8	1.6
Rumen parameters				
pH**	6.68[a]	6.29[b]	6.13[c]	.05
NH_3, m*M**	8.34[b]	10.88[a]	10.46[a]	.64
Total VFA, m*M**	84.41[b]	96.01[a]	100.44[a]	2.53
Molar proportion, %				
Acetic**	75.42[a]	72.29[b]	68.85[c]	.38
Propionic**	14.56[b]	15.06[b]	17.09[a]	.49
Butyric**	6.80[c]	9.12[b]	11.02[a]	.30
I-Butyric	1.12	1.08	1.09	.05
Valeric	1.13	1.09	1.23	.05
I-Valeric plus 2-methylbutyric**	1.30[b]	1.36[b]	1.70[a]	.06

[a,b,c]Means within rows with different superscripts differ ($P<.05$).

[1] Percent forage (F) or concentrate (C).

*Significant effect of diet ($P<.05$).

**Significant effect of diet ($P<.01$).

TABLE 5. Effect of diet on rumen microbial numbers and protein degradation activity.

Item	Diet[1] 100F	63F/37C	37F/63C	SE
Total protozoal count, $\times 10^4$/ml*	8.14[b]	25.77[b]	122.82[a]	12.56
Total bacterial count, $\times 10^9$/ml**	3.36[b]	7.04[a]	10.00[a]	.49
Viable bacterial count, $\times 10^9$/ml**	.98[b]	2.35[a]	3.27[a]	.21
Microbial dry weight, mg/ml**	23.5[c]	34.0[b]	41.2[a]	.92
Microbial N, % DM basis	7.91	7.67	7.87	.19
Degradation rate, mg N/h per g DM				
Casein	2.334	2.045	1.745	.188
BSA[2]	.489	.467	.514	.058
Fractional degradation rate, h^{-1}				
Casein*	.230[b]	.265[a,b]	.293[a]	.013
BSA**	.046[b]	.060[b]	.084[a]	.007

[a,b,c]Means within rows with different superscripts differ ($P<.05$).

[1] Percent forage (F) or concentrate (C).

[2] Bovine serum albumin.

*Significant effect of diet ($P<.05$).

**Significant effect of diet ($P<.01$).

forage diet. Others (12, 24) also have reported a positive relationship between dietary concentrate, butyric acid production, and protozoal numbers. The 100% forage diet yielded the lowest total bacterial count. The 37% forage diet yielded total and viable bacterial counts, which were approximately three times greater than the 100% forage diet. Bacterial viability ranged from 29 to 33% and was not significantly influenced by diet. The microbial dry weight was also greatest on the 37% forage diet. No differences were detected in concentration of nitrogen in microbial pellets indicating that feed particle contamination was not confounding the interpretations based on microbial dry weight. When protein degradation rates were expressed per unit microbial DM, no significant differences were observed for either casein or BSA. However, casein fractional degradation rate was highest for the 37% forage diet, intermediate for the 63% forage diet, and lowest on the 100% forage diet. The BSA fractional degradation rate also was highest on the 37% forage diet.

Dietary energy appears to increase protein degradation mainly through increased microbial numbers or mass. Protein degradative activity per unit microbial mass was increased somewhat for SBM and casein with inocula enriched with particle-associated organisms (Table 3). Nevertheless, protein degradative activity appears to be distributed between the fluid and adherent microbial populations in the rumen. This suggests that protein degradation may be principally a function of factors that influence microbial numbers or mass, such as the dietary energy concentration or fermentability (2).

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REFERENCES

1 Association of Official Agricultural Chemists. 1980. Official methods of analysis. 13th ed. Washington, DC.

2 Bates, D. B., and W. G. Bergen. 1983. Regulation of exoprotease activity in *Bacteroides ruminicola* GA-33. Page 281 *in* XVII Conf. Rumen Function.

3 Brock, F. R., C. W. Forsberg, and J. G. Buchanan-Smith. 1982. Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. Appl. Environ. Microbiol. 44:561.

4 Broderick, G. A. 1978. In vitro procedures for estimating rates of ruminal protein degradation and

proportions of protein escaping the rumen undegraded. J. Nutr. 108:181.
5 Broderick, G. A. 1984. In vitro determination of rates of ruminal protein degradation. Can. J. Anim. Sci. 64(Suppl.):31.
6 Broderick, G. A., and J. H. Kang. 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. J. Dairy Sci. 63:64.
7 Bryant, M. P., and L. A. Burkey. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. 36:205.
8 Caldwell, D. R., and M. P. Bryant. 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. Appl. Microbiol. 14:794.
9 Craig, W. M., G. A. Broderick, and D. B. Ricker. 1987. Quantitation of microorganisms associated with the particulate phase of ruminal ingesta. J. Nutr. 117:56.
10 Craig, W. M., B. J. Hong, G. A. Broderick, and R. J. Bula. 1984. In vitro inoculum enriched with particle-associated microorganisms for determining rates of fiber digestion and protein degradation. J. Dairy Sci. 67:2902.
11 Dehority, B. A., and J. A. Grubb. 1980. Effect of short-term chilling of rumen contents on viable bacterial numbers. Appl. Environ. Microbiol. 39: 376.
12 Eadie, J. M., J. Hyldgaard-Jensen, S. O. Mann, R. S. Reid, and F. G. Whitelaw. 1970. Observations on the microbiology and biochemistry of the rumen in cattle given different quantities of a pelleted barley ration. Br. J. Nutr. 24:157.
13 Forsberg, C. W., and K. Lam. 1977. Use of adenosine-5′-triphosphate as an indicator of the microbiota biomass in rumen contents. Appl. Environ. Microbiol. 33:528.
14 Hazelwood, G. P., C. G. Orpin, Y. Greenwood, and M. E. Black. 1983. Isolation of proteolytic rumen bacteria by use of selective medium containing leaf fraction 1 protein (ribulosebisphosphate carboxylase). Appl. Environ. Microbiol. 45:1780.
15 Leedle Ziegler, J. A., and R. B. Hespell. 1980. Differential carbohydrate media and anaerobic replica plating techniques in delineating carbohydrate-utilizing subgroups in rumen bacterial populations. Appl. Environ. Microbiol. 39:709.
16 Mahadevan, S., F. D. Sauer, J. D. Erfle, R. M. Teather, and P. M. Morse. 1982. Changes in ammonia concentration, bacterial counts, pH and volatile fatty acid concentration in rumen of cows fed alfalfa hay or concentrate: urea-corn silage. Can. J. Anim. Sci. 62:249.
17 Mangan, J. L. 1972. Quantitative studies on nitrogen metabolism in the bovine rumen. Br. J. Nutr. 27:261.
18 McDougall, E. I. 1948. Studies on ruminant saliva. I. The composition and output of sheeps' saliva. Biochem. J. 43:99.
19 Nugent, J.H.A., and J. L. Mangan. 1981. Characteristics of the rumen proteolysis of fraction 1 (185) leaf protein from lucerne (*Medicago sativa* L.). Br. J. Nutr. 46:39.
20 Ottenstein, D. M., and D. A. Bartley. 1971. Separation of free acids C2-C5 in dilute aqueous solution column chromatography. J. Chromatogr. Sci. 9: 673.
21 Senshu, T., K. Nakamura, A. Sawa, H. Miura, and T. Matsumoto. 1980 Inoculum for in vitro rumen fermentation and composition of volatile fatty acids. J. Dairy Sci. 63:305.
22 Smith, R. H., and O. E. Mohamed. 1977. Effect of degradation in the rumen on dietary protein entering the ruminant duodenum. Proc. Nutr. Soc. 36: 153A.
23 Steel, R.G.D., and J. H. Torrie. 1960. Principles and procedures of statistics. McGraw-Hill Book Co., New York, NY.
24 Whitelaw, F. G., J. M. Eadie, S. O. Mann, and R. S. Reid. 1972. Some effects of rumen ciliate protozoa in cattle given restricted amounts of a barley diet. Br. J. Nutr. 27:425.